

Initial Characterization of the Transplant of Immortalized Chromaffin Cells for the Attenuation of Chronic Neuropathic Pain

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Cultures of embryonic day 17 (E17) rat adrenal and neonatal bovine adrenal cells were conditionally immortalized with the temperature-sensitive allele of SV40 large T antigen (tsTag) and chromaffin cell lines established. Indicative of the adrenal chromaffin phenotype, these cells expressed immunoreactivity (ir) for tyrosine hydroxylase (TH), the first enzyme in the synthetic pathway for catecholamines. At permissive temperature in vitro (33°C), these chromaffin cells are proliferative, have a typical rounded chromaffin-like morphology, and contain detectable TH-ir. At nonpermissive temperature in vitro (39°C), these cells stop proliferating and express increased TH-ir. When these immortalized chromaffin cells were transplanted in the lumbar subarachnoid space of the spinal cord 1 week after a unilateral chronic constriction injury (CCI) of the rat sciatic nerve, they survived longer than 7 weeks on the pia mater around the spinal cord and continued to express TH-ir. Conversely, grafted chromaffin cells lost Tag-ir after transplant and Tag-ir was undetectable in the grafts after 7 weeks in the subarachnoid space. At no time did the grafts form tumors after transplant into the host animals. These grafted chromaffin cells also expressed immunoreactivities for the other catecholamine-synthesizing enzymes 7 weeks after grafting, including: dopamine-β-hydroxylase (DβH) and phenylethanolamine-N-methyltransferase (PNMT). The grafted cells also expressed detectable immunoreactivities for the opioid met-enkephalin (ENK), the peptide galanin (GAL), and the neurotransmitters γ-aminobutyric acid (GABA) and serotonin (5-HT). Furthermore, after transplantation, tactile and cold allodynia and tactile and thermal hyperalgesia induced by CCI were significantly reduced during a 2-8-week period, related to the chromaffin cell transplants. The maximal antinociceptive effect occurred 1-3 weeks after grafting. Control adrenal fibroblasts, similarly immortalized and similarly transplanted after CCI, did not express any of the chromaffin antigenic markers, and fibroblast grafts had no effect on the allodynia and hyperalgesia induced by CCI. These data suggest that embryonic and neonatal chromaffin cells can be conditionally immortalized and will continue to express the phenotype of primary chromaffin cells in vitro and in vivo; grafted cells will ameliorate neuropathic pain after nerve injury and can be used as a homogeneous source to examine the mechanisms by which chromaffin transplants reverse chronic pain. The use of such chromaffin cell lines that are able to deliver antinociceptive molecules in models of chronic pain after nerve and spinal cord injury (SCI) offers a novel approach to pain management.

Key words: Chromaffin cells; Cell lines; Transplantation; Chronic constriction injury

INTRODUCTION

Primary chromaffin cells have been used for transplantation and delivery of therapeutic molecules into recipients for a variety of therapeutic indications, including pain, Parkinson's disease, traumatic brain injury, stroke, and depression (63). Secretion of neurotransmitters, neurotrophic factors, opioids, neuropeptides, excitatory amino acid antagonists, or other unknown substances from these cells has been demonstrated to effectively halt or reverse various disease processes (28). Chromaffin cells from both human and bovine sources have been used in early stage clinical trials for

the treatment of Parkinson's disease and chronic pain (77).

However, a serious limitation in the widespread application of this approach for the therapeutic application is the necessity of harvesting fresh cells from donors because chromaffin cells are mostly postmitotic. This approach is costly, time consuming, and inconvenient. In addition, the resultant cell preparation is obtained from different donors, possibly requiring complete safety screening for each batch of cells. In addition, this primary culture approach could result in different mixtures of cell types that are incompletely characterized and nonhomogeneous.

The generation of chromaffin cell lines would overcome many of the limitations of primary chromaffin cell culture. However, past experience in other laboratories has indicated that, with the exception of tumor cell lines such as pheochromocytomas, chromaffin cell lines are difficult to generate. Pheochromocytoma cell lines are inappropriate for therapeutic use due to the great risk of unrestricted cell division and tumor formation (30). Moreover, pheochromocytoma cell lines, such as the rat PC12 line, may not behave as normal mature chromaffin cells, in that they appear to maintain or dedifferentiate to a less mature phenotype. For example, PC12 cells synthesize dopamine and some norepinephrine, but only low levels of epinephrine, a principal catecholamine in mature chromaffin cells (27). In support for this, these cells have also been found to lack detectable levels of phenylethanolamine-*N*-methyltransferase (PNMT), which catalyzes the conversion of norepinephrine to epinephrine (27).

Several attempts have been made to generate immortalized chromaffin cell lines. Birren and Anderson (5) have reported *v-myc* immortalized sympathoadrenal progenitor cell lines from embryonic rat adrenal glands. Suri et al. (75) have generated adrenal cell lines from tyrosine hydroxylase SV40 T antigen transgenic mice. However, like PC12 cells, in both of these cases the immortalized cells generated produced dopamine and some norepinephrine, but not epinephrine, and lacked the synthetic enzyme PNMT.

An additional limitation using both of these approaches is that the immortalized cells generated are oncogenically transformed and continue to divide, creating a risk of tumorigenesis if transplanted into an immune-privileged site such as the central nervous system. An exception to this in the *v-myc* immortalized lines is the differentiation of a small percentage of cells to postmitotic neurons. However, these postmitotic cells become NGF dependent and rapidly die in its absence (5). Conditional immortalization is a means to generate an immortalized cell line that can later be disimmortalized to stop cell division. Several approaches for conditionally immortalizing cell lines have been described in the literature. One approach involves the use of the temperature-sensitive mutant of the SV40 large T antigen (tsTag) (26) and has been used successfully to conditionally immortalize embryonic CNS neurons (23). Using this approach, transfected cells undergo continual cell division at low temperature conditions (e.g., 32–34°C), but differentiate and become postmitotic when the temperature is raised (37–39°C). The host CNS environment to receive these tsTag-immortalized cell lines has a temperature about 36–39°C, and transplantation allows the cells to cease proliferation and continue differentiation with-

out tumor formation. Thus, conditional immortalization with the tsTag construct incorporates the advantages of cell lines, including the convenience of growing large quantities that can be characterized and safety tested and the ability to genetically engineer in additional therapeutic molecules, while reducing the disadvantages of tumor cell lines.

Conditional immortalization of chromaffin cells using tsTag has recently been reported (16,25), and here we describe the use of subarachnoid grafted chromaffin cell lines in reducing pain. The preliminary data describing the use of these grafted cell lines for neuropathic pain have been recently reported (17). This transplantation approach to chronic pain after nerve injury is expected to provide a useful model for the creation of similarly derived human cell lines and their application to a strategy for the alleviation of chronic pain following human peripheral nerve and SCI.

MATERIALS AND METHODS

Primary Culture and Immortalization of Dissociated Rat and Bovine Adrenals

Methods to culture chromaffin cells from rat embryonic adrenal tissue have been previously described (24). The generation and initial *in vitro* characterization of the immortalized rat and bovine cell lines used here for transplantation have been recently described (16,25). Briefly, primary cultures of rat chromaffin cells were established from Sprague-Dawley rat embryos on fetal day 17 (E17) and cultures were grown overnight before infection with immortalizing viruses.

Cultures were incubated overnight with the conditioned medium from the competent retroviral producer line ψ2-tsTag, containing the sequence for tsTag expression and resistance to the antibiotic G418, before selection with the antibiotic G418 (Genticin; GIBCO).

Similarly, primary cultures of chromaffin cells were established from young neonate calf (73). Purification of the cell population was accomplished on a Renografin gradient (Squibb Meyers, New Brunswick, NJ). The band containing viable chromaffin cells was plated and allowed to grow overnight before infection with the retrovirus encoding tsTag. Again, immortalized cells were selected with the antibiotic G418.

Good survival of chromaffin clones required that some fibroblasts were carried through each subcloning, and each final chromaffin line contains a small number of adherent fibroblasts that must be removed by differential plating (16) before use. The rat chromaffin cell line used for transplantation after nerve injury is called RAD5.2; the bovine chromaffin line is called BADA20.

Cell Culture and Transplantation of Rat Chromaffin Clones

The methods for transplantation of similarly immortalized neuronal rat cell lines into the subarachnoid space of the lumbar spinal cord after nerve injury for pain relief has been described previously (15). The chromaffin and fibroblast control cell lines were grown at permissive temperature (33°C) in CNS medium/10% FBS/125 µg/ml G418 as described previously (16,80). Adult female (180 g) Wistar-Furth rats were used for transplantation. The chromaffin and control fibroblasts were proliferated at 33°C to near confluence. Immediately before transplantation, cells were gently dissociated from six-well culture plates with sterile 0.5 mm EDTA/DPBS, pelleted by centrifugation, counted by trypan blue exclusion, and suspended in a concentration of 10⁶ µl of Ca²⁺/Mg²⁺-free Hank's buffered saline solution (CMF-HBSS). Following a partial laminectomy and a small puncture of the dura, cells (10⁶) were injected into the subarachnoid space of the lumbar dorsal spinal cord, by a dorsal/caudal entry into the dural puncture a few millimeters with a small length of polyethylene (PE-10) tubing containing the cells, at spinal segment L1, 1 week after the CCI. For both CCI and transplantation, animals were anesthetized with a mixture of ketamine, xylazine, and acepromazine, 0.65 ml/kg. Animals were allowed to recover at 37°C for 12 h, after which time they were returned to the animal care facility, and housed one per cage with rat chow and water ad lib on a 12/12 h light/dark cycle.

Immunocytochemistry

In a group of animals different than that used to assess sensory behaviors after cell transplants, spinal cords were fixed weekly for survival and immunohistochemical staining, 1–7 weeks after CCI and transplantation, by transcardial perfusion fixation (69). The fixative, consisting of 4% paraformaldehyde, 10% picric acid in DPBS also contained 0.1% glutaraldehyde to localize the various antigens in the grafted cells. After removal from the vertebral column, cords were stored in fix for 12 h, 4°C, before the tissue was processed for paraffin embedding. After embedding, cords were sectioned, 10 µm thick, and sagittal sections collected serially to poly-lysine-coated slides. Slides were cleared for 5 min in xylene, rehydrated, and boiled in citrate buffer, pH 7.4, for 15 min at 65°C before beginning the immunostaining procedures. Sections were stained for bromodeoxyuridine (BrdU), tyrosine hydroxylase (TH), dopamine-β-hydroxylase (DβH), PNMT, γ-aminobutyric acid (GABA), galanin (GAL), or met-enkephalin (ENK) and dehydrated, cleared, and mounted in Permount (Sigma) after antibody staining. Two kinds of negative

controls were used to assess any possible nonspecific staining for each antigen: 1) the same antigens labeled in fibroblasts grafts, and 2) chromaffin grafts stained in the absence of the primary antibody. Grafted chromaffin cells always contained high-intensity signal, compared to the cord; only grafted cells were considered for quantitation.

Rats were euthanized for tissue fixation by a combination of pentobarbital overdose and exsanguination. Animals were anesthetized with an IP injection of sodium pentobarbital (12 mg/100 g). Once the appropriate level of anesthesia was reached (i.e., no corneal or withdrawal reflexes), the rat was transcardially perfused with aldehydes. After perfusion, the spinal cord and transplant were removed and histologically processed. All surgical interventions, pre- and postsurgical animal care, and euthanasia were in accordance with the Laboratory Animal Welfare Act, *Guide for the Care and Use of Laboratory Animals* (NIH, DHEW Pub. No. 78-23, Revised, 1978), and guidelines provided by the Animal Care and Use Committee of the University of Miami, Miami, FL.

BrdU. Cells used for transplants and antibody staining were treated with 10 µm BrdU during proliferation for 3 days before transplantation and grafts were stained for BrdU posttransplantation as described previously (19). The nucleic acid BrdU, followed by a anti-BrdU antibody (Becton Dickenson, Mountain View, CA), has been commonly used (84) to mark cell nuclei and was used here to mark the surviving cells transplanted (68) in the subarachnoid space of the lumbar spinal cord. Briefly, sections were rehydrated in DPBS buffer, followed by 2 N HCl for 30 min and rinsing with DPBS. Sections were then placed in borate buffer for 15 min [borate buffer: borax, 0.95 g/50 ml water and boric acid, 0.625 g/50 ml water (1:1)] and rinsed two times with DPBS. Sections were then permeabilized with 0.02% TX/DPBS for 5 min, followed by a DPBS rinse. Sections were then incubated with the anti-BrdU antibody (1:20 dilution/DPBS) overnight at 4°C, followed by the secondary reporter. The secondary reporter used for BrdU localization was the mouse Elite ABC kit (Vector Labs, Inc., Burlingame, CA) with substitution of the DAB substrate with "VIP" substrate (very intense purple, Vector).

DβH. Some sections were alternately stained for DβH expression to assess whether the chromaffin grafts were able to synthesize norepinephrine during the course of the experiments. Antibody staining for DβH in the grafts is a modification of methods described previously (2). After removal from the vertebral column, cords were stored in fix for 12 h, 4°C, before paraffin embed-

ding and thin sectioning. The anti-D β H antibody (1:1000/DPBS; Chemicon) was incubated with TX-permeabilized (0.2%/DPBS) sections overnight at 4°C, followed by an anti-rabbit peroxidase ABC reporter (Vector) and "VIP" substrate (Vector).

GABA. Methods for staining transplanted cells for GABA have been described previously (19). The anti-GABA antibody (1:5000/DPBS; Incstar) was incubated with TX-permeabilized (0.2%/DPBS) sections overnight at 4°C, followed by an anti-rabbit peroxidase ABC reporter (Vector) and "VIP" substrate (Vector).

GAL. Some sections were also stained for GAL to assess whether the chromaffin grafts were able to synthesize GAL during the course of the experiments. Antibody staining for GAL in the grafts is a modification of methods described previously (6,10,45,46). After removal from the vertebral column, cords were stored in fix for 12 h, 4°C, before paraffin embedding and thin sectioning. The anti-GAL antibody (1:2000/DPBS; pH 7.4; Peninsula Labs-RAS7141) was incubated with TX-permeabilized (0.2%/DPBS) sections overnight at 4°C, followed by an anti-rabbit peroxidase ABC reporter (Vector) and "VIP" substrate (Vector).

ENK. Some sections were also stained for ENK to assess whether the chromaffin grafts were able to synthesize enkephalin during the course of the experiments. Antibody staining for ENK in the grafts is a modification of in vitro methods described previously (16). After removal from the vertebral column, cords were stored in fix for 12 h, 4°C, before paraffin embedding and thin sectioning. The anti-ENK antibody (1:1000/DPBS; Peninsula Labs) was incubated with TX-permeabilized (0.2%/DPBS) sections overnight at 4°C, followed by an anti-rabbit peroxidase ABC reporter (Vector) and "VIP" substrate (Vector).

PNMT. Some sections were also stained for PNMT to assess whether the chromaffin grafts were able to synthesize epinephrine during the course of the experiments. Antibody staining for PNMT in the grafts is a modification of methods described previously (9). After removal from the vertebral column, cords were stored in fix for 12 h, 4°C, before paraffin embedding and thin sectioning. The anti-PNMT antibody (1:1000/DPBS; Incstar) was incubated with TX-permeabilized (0.2%/DPBS) sections overnight at 4°C, followed by an anti-rabbit peroxidase ABC reporter (Vector) and "VIP" substrate (Vector).

Serotonin (5-HT). Methods for staining for 5-HT were modified from those described elsewhere (20). Briefly, sections of lumbar spinal cord with transplants were permeabilized with 0.2% TX/DPBS for 10 min. The anti-5HT antibody (1:5000; Incstar) was incubated with permeabilized sections overnight at 4°C, followed

by an anti-rabbit peroxidase ABC reporter (Vector) and "VIP" substrate (Vector).

Tag. In another set of grafted animals, sections were stained at various time points after transplant of rat and bovine chromaffin cell lines to determine if grafts continued to express Tag-ir at nonpermissive temperature in vivo, which would reflect the possibility of continued proliferation. Antibody staining for Tag in the grafts is a modification of the methods described for in vitro staining for Tag in the chromaffin cell lines (16) and in vivo in cell lines with the Tag construct (21). After CCI and grafting RAD5.2 and BADA.20 chromaffin cells, cords were collected at 3 and 7 days and 7 weeks after transplant. After removal from the vertebral column, cords were stored in fix for 12 h, 4°C, before routine paraffin embedding and thin sectioning (10–15 μ m). Slides were cleared for 5 min in xylene, rehydrated, and boiled in citrate buffer, pH 7.4, for 15 min at 65°C before beginning the immunostaining procedures. Sections were rehydrated in DPBS and incubated in blocking buffer for 30 min (0.4% TX/5% NGS/DPBS). The anti-Tag antibody (the cell-conditioned medium diluted 1:1 with the blocking buffer) from the hybridoma PB101, clone 412 (ATCC) was incubated with the sections overnight at 4°C, followed by an anti-rabbit Oregon green fluorescent reporter (1:150 dilution in blocking buffer; Molecular Probes) for 1 h, which included 1 μ M bis benzamide dye, to stain viable nuclei. Sections were mounted in no-fade and stored at –20°C until photographed on a Zeiss Axiophot microscope, on Elite Ektachrome 400 slide film. The images were scanned to ZIP format and arranged in Adobe Photoshop.

TH. Rat spinal cords were fixed weekly for survival and TH staining, 1–6 weeks after CCI and transplantation, by transcardial perfusion fixation by methods described elsewhere (69). Antibody staining for TH in the grafts is a modification of methods described previously (50,74). After removal from the vertebral column, cords were stored in fix for 12 h, 4°C, before paraffin embedding and thin sectioning. The anti-TH antibody (1:5000/DPBS; Chemicon) was incubated with TX-permeabilized (0.2%/DPBS) sections overnight at 4°C, followed by an anti-rabbit peroxidase ABC reporter (Vector) and "VIP" substrate (Vector). Every second section was stained for BrdU or TH and dehydrated, cleared, and mounted in Permamount after antibody staining.

Quantitation of Antigens in Chromaffin Grafts

Spinal cords were removed 7 weeks after grafting and nerve injury and stained for the antigens as described above. Sections that were immunopositive for BrdU defined the lateral extent(s) of the graft locations, and 110–130 sections were usually used from each cord to

identify grafted, labeled cells. Grafted cells were labeled in serial alternative sections. BrdU-ir cells were counted in every third section, TH in every fourth section, GABA in every fifth section, 5-HT in every sixth section, ENK in every seventh section, and GAL in every eighth section. For each type of chromaffin cell graft, rat and bovine cells, spinal cords from three rats were stained and quantified for the presence of antigens in the graft. The data represent the mean of total number of cells expressing each antigen and the mean number of cells expressing each antigen per section.

To measure the spread of grafted cells at 7 weeks after transplant, three to six sections from the epicenter of the transplant, labeled with BrdU-ir, were chosen from each of the three sectioned cords, from each of the chromaffin cell line grafts. The extent of the graft was measured, in a video-captured image at low magnification, with the calibrated "measure function" of the Meta-Morph image analysis software. Data are expressed as the mean \pm SEM of three to six sections from three animals from each type of chromaffin graft.

Chronic Constriction Injury (CCI)

The surgery to produce CCI was first described by Bennet and Xie (4). This model of injury has been used to test the effects of transplants to relieve pain-related behaviors (61), and was subsequently used before transplantation of chromaffin and control cells (15). Under ketamine/xylazine anesthesia, the right common sciatic nerve was exposed at the level of the middle thigh by blunt dissection through the biceps femoris. Proximal to the nerve's trifurcation, a 5–7-mm length of nerve was freed of adhering tissue and four ligatures (4.0 chromic gut) were tied loosely around it with about 1-mm spacing. Care was taken to tie the ligatures so that the diameter of the nerve was barely constricted. The incision was closed in layers and the entire surgery was repeated, minus the ligatures, on the left side to create a sham-operated nerve.

Behavioral Testing

Testing Protocol. One week before CCI and transplants, animals were acclimated and trained for 3 days on all behavioral tests, followed by a baseline measure of the tests described below 1 week before CCI. One week following CCI and every week thereafter animals were retested. Animals to be transplanted were then injected with either the control fibroblast or rat or bovine chromaffin cells. A third group of animals received CCI but no transplants and served as the CCI-alone control group. A fourth group of animals received neither CCI nor transplants and served as the naive control animals. Behavioral testing was repeated for all animals once a week for 8 weeks following CCI and transplants. All

animals used for behavioral testing were sacrificed after 8 weeks of testing.

Another group of animals used to examine survival of cells after CCI and transplantation was sacrificed weekly and was not included with those used for behavioral testing.

Cold Allodynia. Methods for testing the response to cold stimuli have been described elsewhere (4). Each rat was placed under a transparent plastic cover on a cold copper plate ($4 \pm 1^\circ\text{C}$). After 5 min of adaptation, the number of hindpaw lifts for both right (ligated) and left (sham-operated) paw during a 20-min interval was counted. The total duration of hindpaw lifts for each paw over the 20-min interval was recorded. For each session, for each animal, the score of the sham-operated paw was subtracted from the ligated paw. From these values, a mean difference score for each session was calculated for each group of animals and used to determine the effects of the different treatments. Responses were measured once weekly before and after surgery and transplantation.

Thermal Hyperalgesia. Methods for testing thermal hyperalgesia with a Hargreaves device have been described elsewhere (4,29). Animals were placed in a clear Plexiglas box on an elevated Plexiglas floor. Animals were allowed to acclimate for approximately 5 min. A constant intensity, radiant heat source was aimed at the midplantar area of the ligated and sham-operated hind paws. The time, in seconds, from initial heat source activation until paw withdrawal was recorded. Five minutes were allowed between stimulations. Five latency measurements for each paw were recorded at weekly sessions before and after CCI and after transplantation. For each of the five latency measurements, the score of the sham-operated paw was subtracted from the ligated paw. From these values, a mean difference score for each session was calculated for each experimental animal and used to determine the effects of the different treatments.

Mechanical Allodynia. Mechanical allodynia, the occurrence of foot withdrawal in response to normally innocuous mechanical stimuli, was tested using a graded series of von Frey hairs (11). Animals were placed in a Plexiglas box with an elevated mesh floor. After the animal was acclimated for 5 min, calibrated von Frey hairs with ranges from 0.41 to 8.5 g were applied perpendicular to the midplantar area of the ligated and sham-operated hindpaw and depressed slowly until bent. The value, in grams, for the minimal, initial hindpaw withdrawal was recorded for each of five trials. A single trial of stimuli consisted of five applications of a von Frey filament within a 10-s period, to ensure that the response was constant. Each session consisted of five trials, repeated at 3-min intervals on each hindpaw. This mini-

mum value for initial response for the sham-operated paw was subtracted from the score for the ligated paw and all five replicate scores were averaged. From these values, a difference score was calculated and used to determine the effects of the different treatments.

Mechanical Hyperalgesia. Mechanical pain thresholds to noxious stimulation (paw pinch) after cell transplants (56,62) and CCI (4) were determined with a modified version of the Randall-Selitto method (52), using a paw pinch analgesymeter (Stoelting, Wood Dale, IL). A conical stylus with a hemispherical tip (1.2-mm radius) was placed on the middle of the hindpaw dorsal surface. The animal was restrained gently between cupped hands and calibrated pressure of gradually increasing intensity (16 g/s) was applied until the rat withdrew the hind paw. The hind paws were tested alternately at 3-4-min intervals. Five measurements were taken for each side, averaged, and difference score computed by subtracting the average of the control side from the average of the ligated side. From these values, a mean difference score for each session was calculated for each experimental animal and used to determine the effects of the different treatments.

Chemicals

Primary antisera against the indicated antigens were obtained from the following sources: BrdU (Becton Dickinson, Mountain View, CA); D β H (Chemicon, Temecula, CA); GABA (Incstar, Stillwater, MN); GAL (Peninsula Labs Inc., Belmont, CA); Tag (hybridoma PB101, clone 412) (American Type Culture Collection, Rockville, MD); ENK (Peninsula Labs Inc.); PNMT (Incstar); 5-HT (Incstar); and TH (Chemicon). CNS medium (41) and geneticin (G418) were obtained from GIBCO (Grand Island, NY). TCM serum replacement constituent was from Celox Labs, and all other powdered media, attachment factors, and chemicals were purchased from Sigma Chemical (St. Louis, MO). Bovine serum albumin (BSA; fraction V, protease-free) was obtained from Boehringer Mannheim (Indianapolis, IN), and fetal bovine serum (FBS) was from Hyclone (Logan, UT).

Statistical Analysis

Statistical significance of all quantitative data was determined with a multivariate analysis of variance (MANOVA). Comparisons of differences between individual means were tested using the Tukey honest significant differences (HSD) method or the unequal N least significant difference (LSD) test. All of the analyses were performed with a commercially available software package (Statistica, Statsoft, 1990). Values of $p < 0.01$ were considered statistically significant.

RESULTS

Chronic Survival and TH Immunoreactivity in Grafted Cells After Transplantation

Chronic survival of transplanted cells was evaluated in rat and bovine chromaffin cell grafts with anti-BrdU immunohistochemistry at weekly intervals after transplant placement. Examples of transplant survival using BrdU-ir 7 weeks after grafting is seen in Figure 1(A, C, E, G). Colocalization of TH-ir (Fig. 1B, D, F, H) in alternate sections through the grafts demonstrated that surviving transplanted cells continued to express the rat (Fig. 1B) and bovine (Fig. 1D) chromaffin cell phenotype throughout the postgraft period, while sensory behaviors were evaluated. Many cell nuclei positive for BrdU-ir in both cell types were found throughout the 7 weeks after transplant (data not shown). Both types of grafted chromaffin cells attached to the spinal pial surface near the transplant site and were found peripherally several segments rostral and caudal to the L1-2 injection site. However, while many BrdU-ir control fibroblast cells were found after transplant (Fig. 1E, G), they did not contain detectable TH-ir at any time through 7 weeks after transplant (Fig. 1F, H). The rat (Fig. 1B) and bovine (Fig. 1D) chromaffin cell graft sites always contained many TH (+) cells on the pial surface.

Time Course of the Loss of Expression of Tag After Transplant of Chromaffin Cell Lines

The temperature of rat CNS approximates (40) the nonpermissive temperature conditions (about 39°C) for the tsTag-immortalized chromaffin cell lines, and Tag protein is labile under those conditions, is not able to drive mitosis in cells immortalized with the tsTag construct, and neuronal differentiation is favored after transplantation *in vivo*. These temperature-sensitive (ts) cell lines have been successfully transplanted in the CNS without the complications of tumor formation or immunological rejection (70). Immunohistochemistry for Tag was used to examine the chromaffin cell grafts at various time points to assess whether the cell lines had lost Tag-ir and were likely to be differentiating, rather than continuing to proliferate. The disappearance of Tag-ir in the chromaffin grafts is illustrated in Figure 2. Both rat (Fig. 2A-D) and bovine grafts (Fig. 2E-H) were stained for Tag-ir at 3 days (Fig. 2A, E), 1 week (Fig. 2B, F), and 7 weeks (Fig. 2C, G) after grafting at 1 week following CCI. Similar to the loss of Tag-ir in these chromaffin cells during differentiation at nonpermissive temperature *in vitro* (16), Tag-ir disappeared to very low levels and was barely detectable 7 weeks after grafting. Intermediate levels of Tag-ir were visible 7 days after grafting, compared to very high Tag-ir (most of the cells) at 3 days after transplant. The 7-week chromaffin

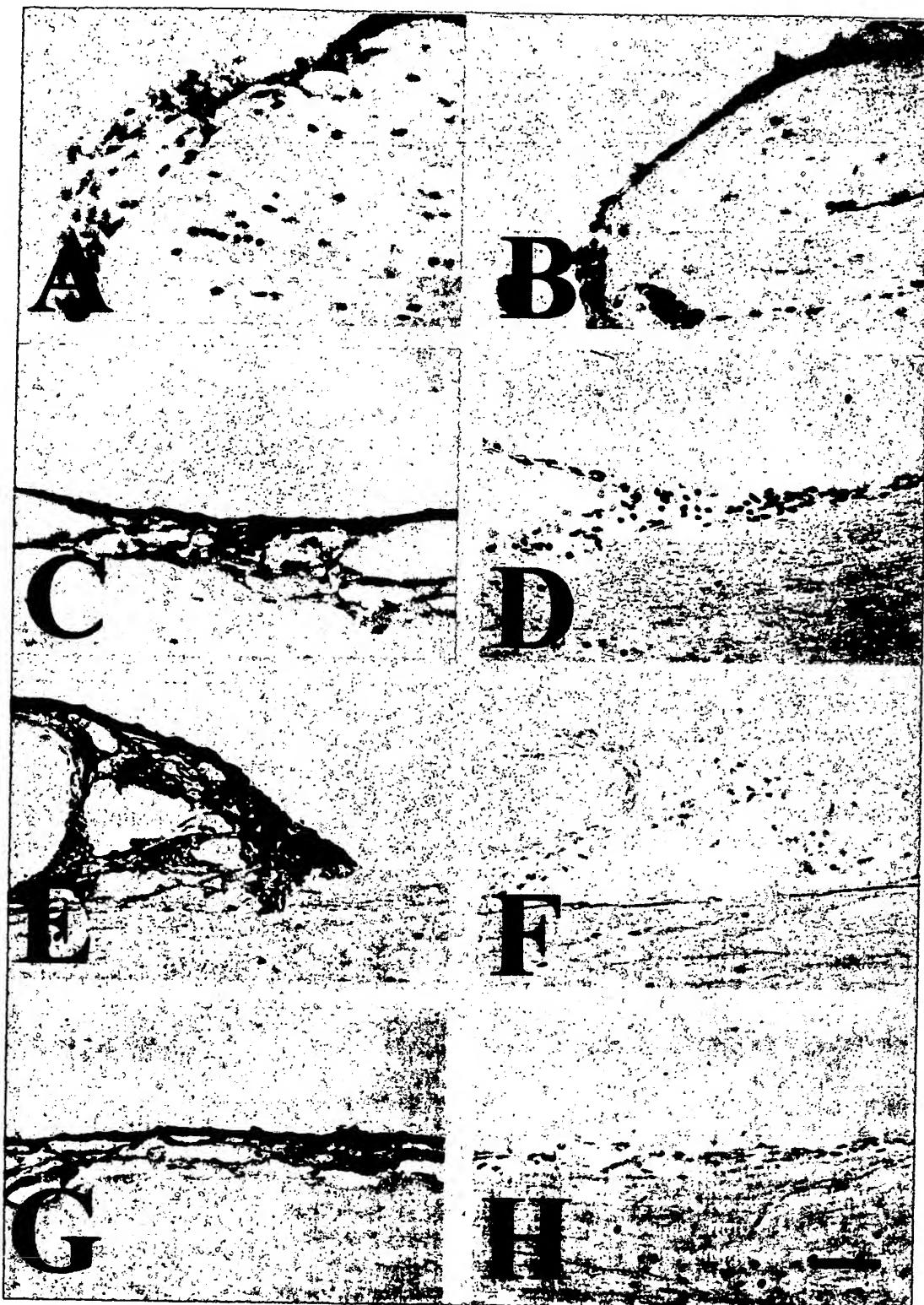


Figure 1. Survival and TH immunoreactivity in transplanted cell lines after nerve injury. The rat RAD5.2 (A, B) and bovine BADA.20 (C, D) chromaffin cells, as well as rat (E, F) and bovine (G, H) fibroblasts were transplanted 1 week after CCI in the lumbar subarachnoid space and examined 7 weeks after transplant for expression of TH-ir. All cells were preincubated before transplant with BrdU to identify surviving cells in the grafts. Sagittal sections were immunohistochemically stained for BrdU-ir (A, C, E, G) and TH-ir (B, D, F, H) in the grafted cells. Only rat RAD5.2 (B) and bovine BADA.20 (D) chromaffin cell grafts contained surviving cells that were both BrdU (+) and TH (+); rat (F) and bovine (H) fibroblast grafts had no detectable TH-ir, even though many BrdU (+) fibroblast cells (E, G) survived >7 weeks after transplant and nerve injury. Bar = 100 μ m.

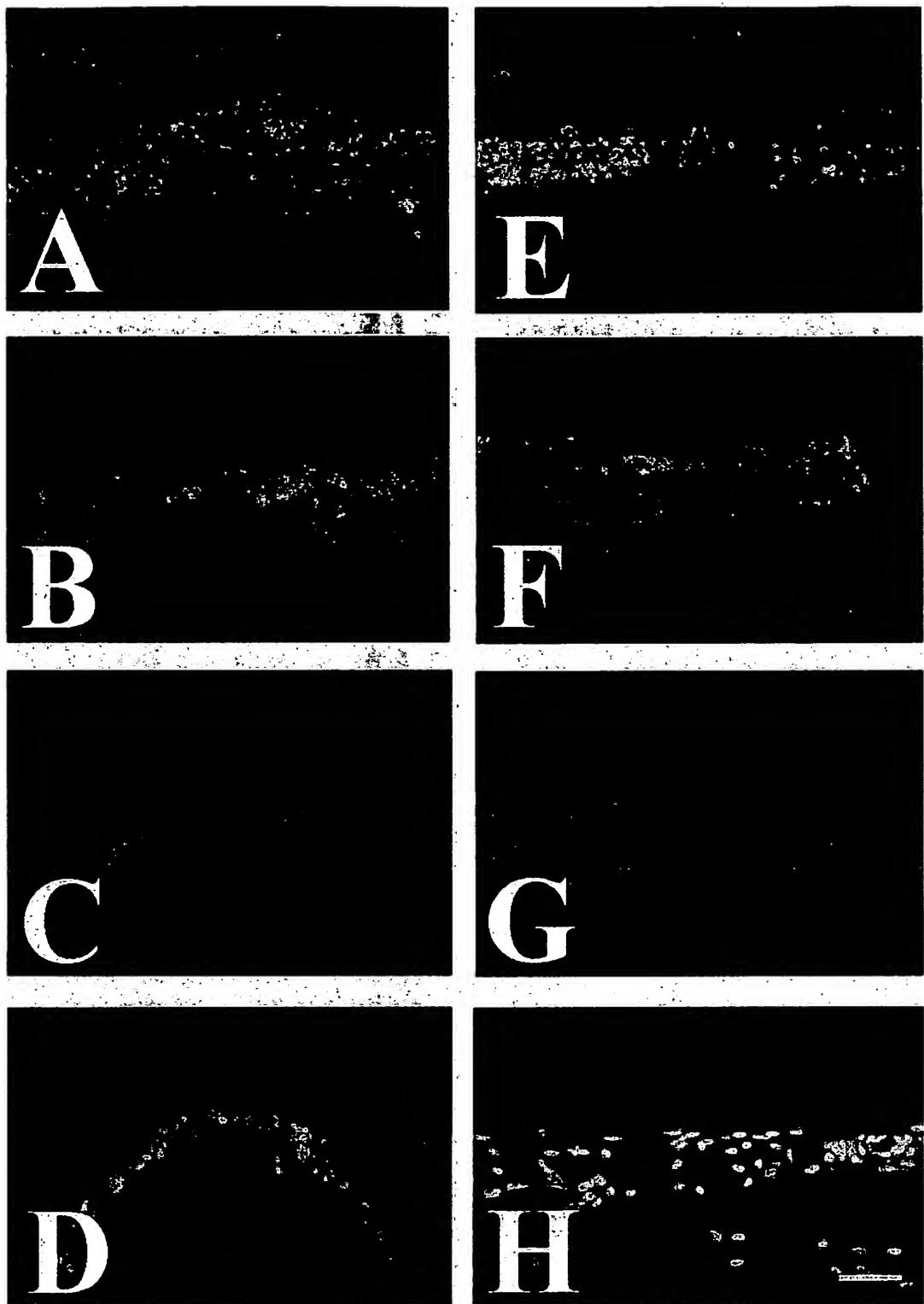


Figure 2. Loss of Tag immunoreactivity in chromaffin cell transplants. Rat (A-D) and bovine (E-H) chromaffin cells were transplanted 1 week after CCI, and spinal cords removed after 3 (A, E) and 7 (B, F) days and 7 weeks (C, D, G, H) after graft placement. Sagittal sections were examined for Tag-ir and counterstained with the nuclear dye bis benzamide (D, H) to identify viable grafted cells. Although most transplanted chromaffin cells stained for Tag-ir at 3 days, after 7 days Tag-ir is very reduced in both rat (B) and bovine (F) chromaffin grafts. However, 7 weeks after transplant Tag-ir is not detectable in either rat (C) or bovine (G) chromaffin grafts. The same 7-week grafts contained many viable cells, as evidenced by the Hoechst nuclear counterstain in the rat (D) and bovine (H) chromaffin transplant. Bar = 200 μ m.

grafts examined for Tag-ir, however, remained viable at this time point, as evidenced by nuclear counterstaining with bis benzamide (Fig. 2D, H).

D_BH and PNMT Enzyme-ir in Grafted Cells After Transplantation

Transplanted chromaffin cells were evaluated in rat and bovine grafts with anti-D_BH and PNMT immunohistochemistry at weekly intervals after transplant placement. Examples of expression of immunoreactivity for these enzymes in chromaffin grafts was seen 7 weeks (Figs. 3 and 4) after grafting. The rat (Fig. 3) and bovine (Fig. 4) chromaffin cell graft sites always contained many DH-ir (Figs. 3A and 4A) and PNMT-ir (Figs. 3B and 4B) cells on the pial surface. Similar surviving grafts of fibroblasts never contained detectable D_BH or PNMT (data not shown).

Other Antigens Expressed by Grafted Chromaffin Cells After Transplantation

Other likely antinociceptive molecules expressed by primary chromaffin cells, such as ENK (3,43), GAL (55), GABA (37), and 5-HT (8,32–34) were examined immunohistochemically after transplant of chromaffin cell lines 7 weeks after transplant and are illustrated in the grafts of rat (Fig. 3) and bovine (Fig. 4) chromaffin cells. Again, grafts of chromaffin cell lines were initially located with anti-BrdU immunohistochemistry and alternate sections stained for ENK-ir (Figs. 3F and 4F), GAL-ir (Figs. 3E and 4E), GABA-ir (Figs. 3D and 4D), and 5-HT-ir (Figs. 3C and 4C) in the grafts. Both types of chromaffin grafts contained detectable ENK-, GAL-, GABA-, and 5-HT-ir, while fibroblasts contained no detectable levels of these markers (data not shown).

Quantitation of Survival and Phenotypic Markers in Grafted Chromaffin Cells After Transplantation

Immunoreactivity for the catecholamine enzymes and other chromaffin phenotype markers illustrated above were quantified in chromaffin cell grafts 7 weeks after transplant and nerve injury, and the results are summarized in Table 1 (bovine grafts) and Table 2 (rat grafts). After examination of cords that received a transplant of either rat or bovine chromaffin cells, approximately 2% of injected (10^6) cells marked by BrdU survived to the end of the experiment. Most, about 60%, of the bovine cells and 63% of the rat cells continued to express TH. Other inhibitory, antinociceptive markers were expressed in smaller numbers of rat and bovine grafted cells at 7 weeks after transplant and nerve injury. The expression of the phenotype for the neurotransmitters 5-HT and GABA represented 34% and 34%, respectively, of surviving bovine chromaffin grafted cells. In rat chromaffin grafts, the 5-HT phenotype is represented by 31%, and

29% of the cells expressing the GABA phenotype after 7 weeks. The other antigens, ENK and GAL, are expressed by 34% and 31%, respectively, of bovine chromaffin cells; greater than 26% and 26% of the grafted rat chromaffin cells are labeled for ENK- and GAL-ir, respectively, 7 weeks after transplant. The amount of dispersal from the graft site after 7 weeks is also similar in both types of transplants. Although an occasional cell (BrdU positive) was found greater than three spinal segments away from the L1 transplant site, grafted cells extended about 1–3 mm from the graft site (1.28 ± 0.27 mm for rat chromaffin; 2.45 ± 0.45 mm for bovine chromaffin cells) after 7 weeks. There was no significant difference between the two transplant types (rat and chromaffin cells) with regard to numbers of surviving cells (BrdU) or the numbers of cells that expressed the various antigens.

Thermal Hyperalgesia After Transplants

The measure of sensitivity to noxious heat in animals after nerve injury and rat and bovine cell transplants is shown in Figure 5(A, B). In control animals without surgery or transplants, no difference was observed between operated and unoperated hindlimbs in the latency of withdrawal over 8 weeks. The range of latency scores was 6.3–16.9 s in the right hindpaw and 6.1–17.6 s in the left hindpaw at 2 weeks in control animals. After CCI, a vigorous hypersensitivity to heat was observed in the ligated paw 1 week after CCI that was maximal at 2–3 weeks, and did not completely recover by 8 weeks. At 2 weeks after CCI, the range of latency scores was 5.0–10.9 s in the ligated (right) hindpaw and 6.5–18.2 s in the nonligated (left) hindpaw. A similar sensitivity was seen in the ligated paw before transplantation of rat or bovine chromaffin cells at 1 week after CCI. However, 1 week after transplant of rat or bovine chromaffin cells (2 weeks after CCI), the sensitivity in the ligated hindpaw completely disappeared. The range of latencies was 6.8–16.8 s in the ligated hindpaw and 7.0–16.5 s in the nonligated hindpaw at 2 weeks for after transplant of rat chromaffin cells. The range of latencies was 7.1–16.0 s in the ligated hindpaw and 7.2–16.5 s in the nonligated hindpaw at 2 weeks for after transplant of bovine chromaffin cells. After transplant of rat or bovine fibroblast cells, the hypersensitivity observed after CCI in the ligated hindpaw remained for 8 weeks after the CCI. The range of latency scores at 1 week after transplant of fibroblast cells was 4.1–11.1 s in the ligated hindpaw and 6.8–18.1 s in the nonligated hindpaw for rat fibroblast grafts. The range of latency scores at 1 week after transplant of fibroblast cells was 4.3–11.0 s in the ligated hindpaw and 6.8–18.3 s in the nonligated hindpaw for bovine fibroblast grafts.

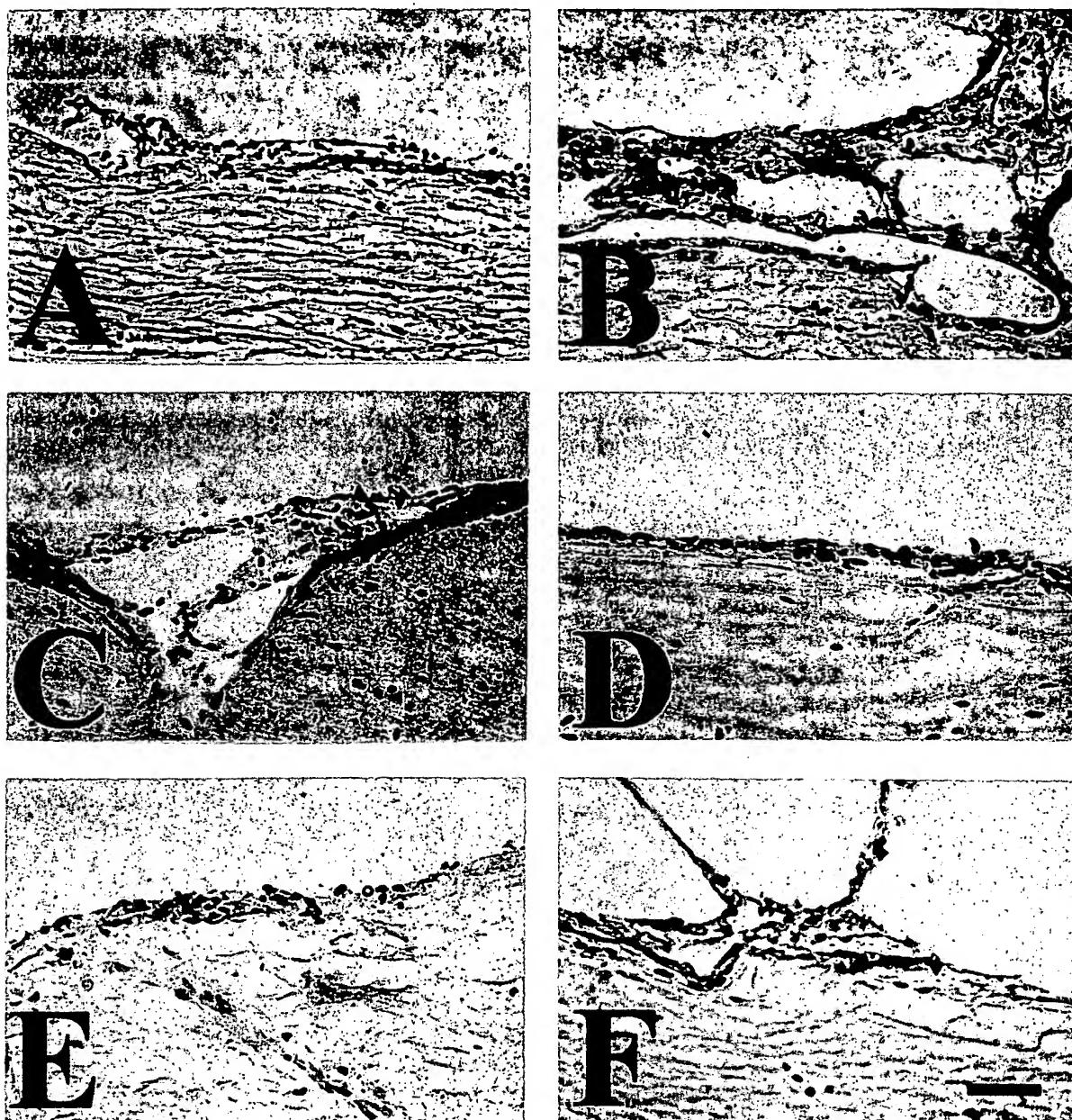


Figure 3. Antigens expressed by rat chromaffin grafts 7 weeks after transplant. The rat RAD5.2 chromaffin cells were transplanted 1 week after CCI and grafts examined 7 weeks later for D β H-ir (A), PNMT-ir (B), 5-HT-ir (C), GABA-ir (D), GAL-ir (E), and ENK-ir (F). All these antigens were easily detectable in the chromaffin cell grafts; rat fibroblast control grafts contained no detectable levels of immunoreactivities (data not shown). Bar = 100 μ m.

Tactile Hyperalgesia After Transplants

The effects of transplants of rat and bovine chromaffin and fibroblast cells on tactile hyperalgesia, or paw pinch, is shown in Figure 6(A, B). Naive animals demonstrated a similar sensitivity in each hindpaw, with the range of latency scores 8.0–13.0 in the left paw and

7.0–15.0 in the right paw. One week after CCI only, the maximum response to paw pinch was seen in the ligated hindpaw and this level of hypersensitivity continued throughout the 8 weeks after CCI. The range of raw latency scores at 1 week after CCI for the ligated hindpaw was 4.0–7.0 and 8.0–15.0 in the nonligated hindpaw. The rat chromaffin cells, placed 1 week after CCI, began

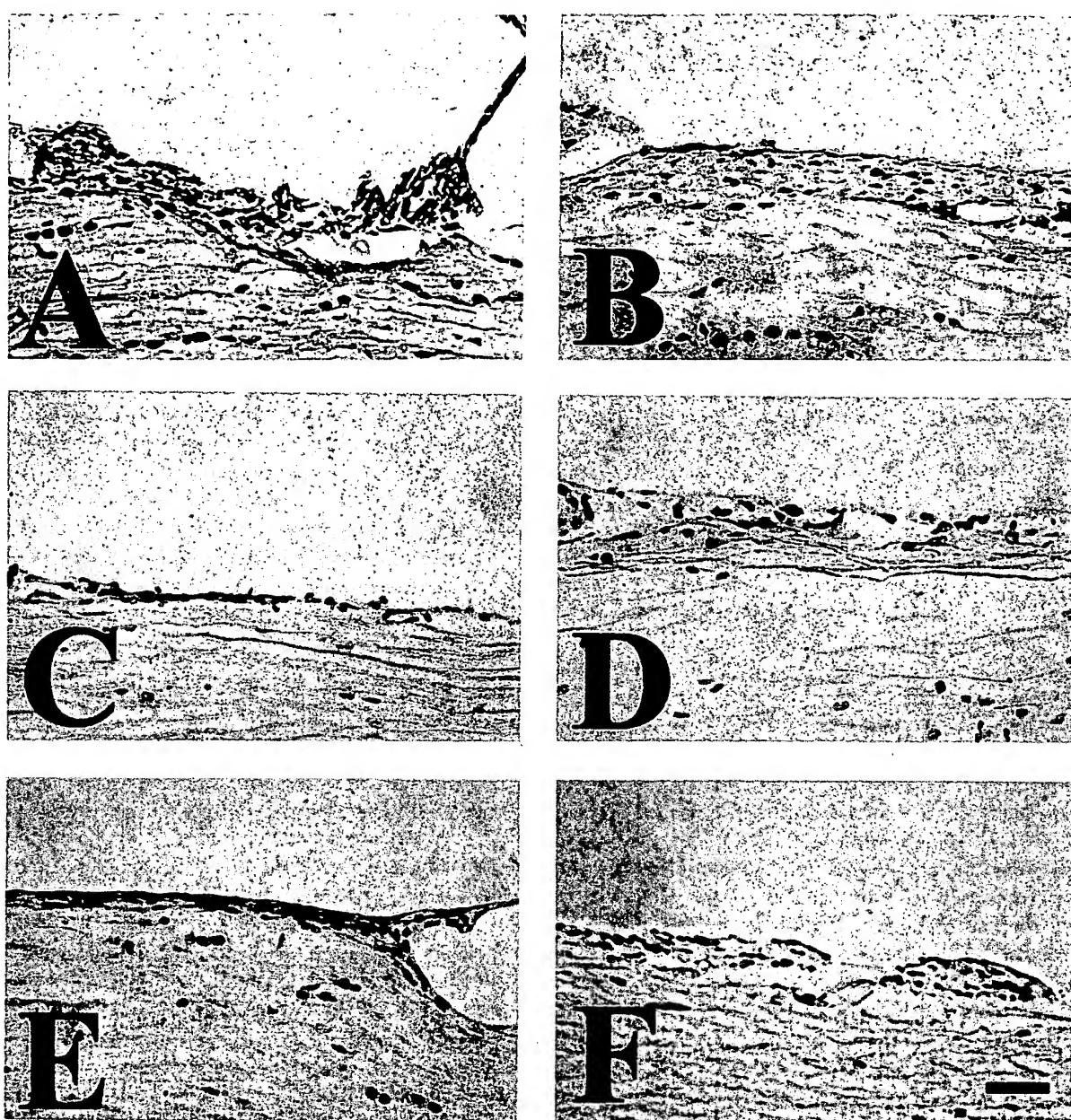


Figure 4. Antigens expressed by bovine chromaffin grafts 7 weeks after transplant. The bovine BADA.20 chromaffin cells were transplanted 1 week after CCI and grafts examined 7 weeks later for D β H-ir (A), PNMT-ir (B), 5-HT-ir (C), GABA-ir (D), GAL-ir (E), and ENK-ir (F). All these antigens were easily detectable in the chromaffin cell grafts; bovine fibroblast control grafts contained no detectable levels of immunoreactivities (data not shown). Bar = 100 μ m.

to reverse the nociception in the ligated hindpaw 1 week after transplantation, with the hindpaw developing a hypersensitivity to paw pinch. At 2 weeks after transplant the range of raw latency scores was 8.0–14.0 for the ligated paw and 7.0–15.0 for the nonligated hindpaw. The bovine chromaffin cells, placed 1 week after CCI, began to reverse the nociception in the ligated hindpaw 1–2 weeks after transplantation, with the maximum ef-

fect at 3 weeks after transplant. At 2 weeks after transplant the range of raw latency scores was 8.4–14.0 for the ligated paw and 8.5–15.0 for the nonligated hindpaw. In contrast, after transplantation of fibroblast cells, the hypersensitivity to paw pinch was not reversed throughout the 8-week course of the experiment. At 2 weeks after transplant of rat fibroblasts, the raw latency scores were 3.0–7.0 for the ligated hindpaw and 9.0–

Table 1. Quantitation of Bovine Chromaffin Grafted Cells

	BrdU	TH	GABA	5-HT	ENK	GAL
Mean total No. of cells \pm SEM/graft	23,906 \pm 110.5	14,174 \pm 87.6	8,112 \pm 66.4	8,104 \pm 51.0	8,253 \pm 47.9	7,410 \pm 57.1
Mean No. cells \pm SEM/section	213 \pm 9.3	126 \pm 12.6	72.4 \pm 6.1	72.4 \pm 5.9	73.7 \pm 4.9	66.0 \pm 5.6

Seven weeks after placing subarachnoid grafts of bovine chromaffin cells, the BADA.20 cells, which had been pretreated with 1 μ M BrdU for 3 days *in vitro* during proliferation at 33°C before transplant, spinal cords were removed, and stained for BrdU, TH, GABA, 5-HT, ENK, and GAL. Only animals that demonstrated tactile and thermal allodynia and hyperalgesia 1 week after CCI were transplanted, and subsequently used for immunohistochemical evaluation. The data represent total number of cells per graft \pm SEM stained for each antigen and the mean number per section \pm SEM from three animals.

15.0 in the nonligated hindpaw. At 2 weeks after transplant of bovine fibroblasts, the raw latency scores were 2.5–8.0 for the ligated hindpaw and 10.0–15.0 in the nonligated hindpaw.

Tactile Allodynia After Transplants

The effects of transplants of the rat and bovine chromaffin and fibroblast cells on mechanical allodynia after CCI are shown in Figure 7(A, B). All animals were examined 1 week before CCI and for 8 weeks after CCI for foot withdrawal in response to stimulation with a graded series of von Frey hairs as described in Materials and Methods. A significant response appeared in the ligated hindpaw 1 week after CCI alone did not resolve during the 8 weeks after surgery. The near maximum nociceptive effect appeared in the ligated paw versus the nonligated paw at 1 week following CCI, with the range of raw latency scores of 0.025–0.675 g for the ligated paw and 1.22–3.55 g in the nonligated paw. At the same time period, the scores for the uninjured control animals varied from 1.20 to 3.60 g in either paw. However, 1 week after rat chromaffin cells were transplanted near the lumbar spinal cord after CCI, the mechanical sensitivity began to resolve, with near normal absence of hypersensitivity by 8 weeks, compared to CCI alone. At this time period, the range of raw latency scores was 0.49–3.60 g in the ligated paw and 3.90–4.08 g in the nonligated paw. Three weeks after bovine chromaffin cells were transplanted near the lumbar spinal cord after CCI, the mechanical sensitivity began to resolve, with near normal absence of hypersensitivity by 8 weeks,

compared to CCI alone. At this time period, the range of raw latency scores was 0.50–3.60 g in the ligated paw and 3.90–4.08 g in the nonligated paw. Transplants of the rat and bovine fibroblast cells had no significant effect on the induction of allodynia by CCI. At this same time period, 2–3 weeks after transplants of rat fibroblast cells, 3–4 weeks after CCI, the range of raw latency scores in the ligated paw was 0.030–0.69 g in the ligated paw and 1.20–3.60 g in the nonligated paw. Two to 3 weeks after transplants of bovine fibroblast cells, 3–4 weeks after CCI, the range of raw latency scores was 0.033–0.72 g in the ligated paw and 1.25–3.25 g in the nonligated paw.

Cold Allodynia After Transplants

The effects of transplants of the rat (Fig. 8A1, B1) and bovine (Fig. 8A2, B2) chromaffin and fibroblast cells on the frequency (Fig. 8A1, A2) and duration (Fig. 8B1, B2) of cold allodynia after CCI are shown in Figure 8. A measure of cold allodynia using hindpaw withdrawal to a cold plate demonstrated a vigorous hypersensitivity 1 week after the CCI alone that continually increased throughout 8 weeks after CCI, and never returned to baseline. At 2 weeks after CCI, the range of latency scores was 10–47 hindpaw lifts, with a 12.5–80.0-s duration in the ligated hindpaw, for the CCI animals in Figure 8A1 and B1. At 2 weeks after CCI, the range of latency scores was 10–36 hindpaw lifts, with 12.5–80.1-s duration in the ligated hindpaw, for the CCI animals in Figure 8A2 and B2. Both the number of hindpaw lifts (Fig. 8A1, A2) and total duration of hindpaw

Table 2. Quantitation of Rat Chromaffin Grafted Cells

	BrdU	TH	GABA	5-HT	ENK	GAL
Mean total No. of cells \pm SEM/graft	20,153 \pm 132.4	12,825 \pm 66.9	5,816 \pm 53.7	6,125 \pm 55.6	5,360 \pm 77.0	5,289 \pm 44.7
Mean No. cells \pm SEM/section	163 \pm 9.1	104 \pm 5.1	47.3 \pm 6.9	49.8 \pm 5.4	43.6 \pm 3.8	43.0 \pm 2.86

Seven weeks after placing subarachnoid grafts of rat chromaffin cells, the RAD5.2 cells, which had been pretreated with 1 μ M BrdU for 3 days *in vitro* during proliferation at 33°C before transplant, spinal cords were removed, and stained for BrdU, TH, GABA, 5-HT, ENK, and GAL. Only animals that demonstrated tactile and thermal allodynia and hyperalgesia 1 week after CCI were transplanted, and subsequently used for immunohistochemical evaluation. The data represent total number of cells per graft \pm SEM stained for each antigen and the mean number per section \pm SEM from three animals.

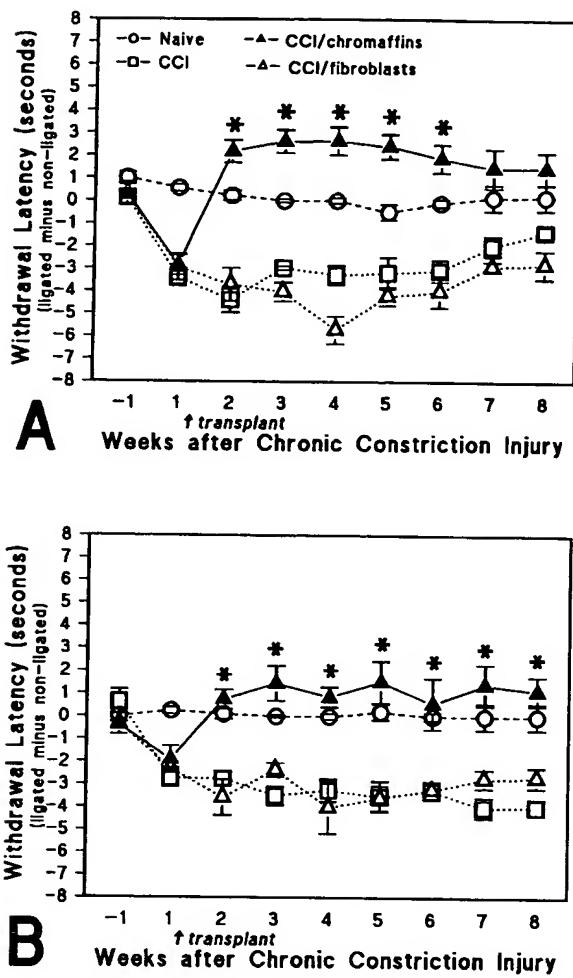


Figure 5. Thermal hyperalgesia after CCI and transplants. Animals were either left unoperated (○), given the CCI (□), or transplanted with rat RAD5.2 (A), bovine BADA20 (B) (▲), or rat (A) or bovine (B) fibroblast cells (Δ) 1 week following the CCI, 1 day following behavioral testing. Animals were tested for hindpaw withdrawal once every week for 1 week before and 8 weeks following CCI and before and after transplants. Only animals that demonstrated thermal hyperalgesia 1 week after CCI were transplanted. The data reported are the mean \pm SEM of the difference values for ligated paw minus the sham-operated paw of 14 animals in each group. Asterisks (*) indicate the chromaffin cell line transplants that differed significantly from the CCI condition at each time point: $p < 0.01$.

withdrawal (Fig. 8B1, B2) were significantly reduced after transplantation of rat and bovine chromaffin cells near the lumbar spinal cord, with the reduction of sensitivity beginning 1 week after transplant of chromaffin cells (2 weeks after CCI). The range of raw latency scores at this time point was 2–8 hindpaw lifts in the ligated paw, with no lifts in the nonligated paw, for the animals in Figure 8A1 and 2–9 hindpaw lifts in the ligated paw, for the animals in Figure 8A2. At the same

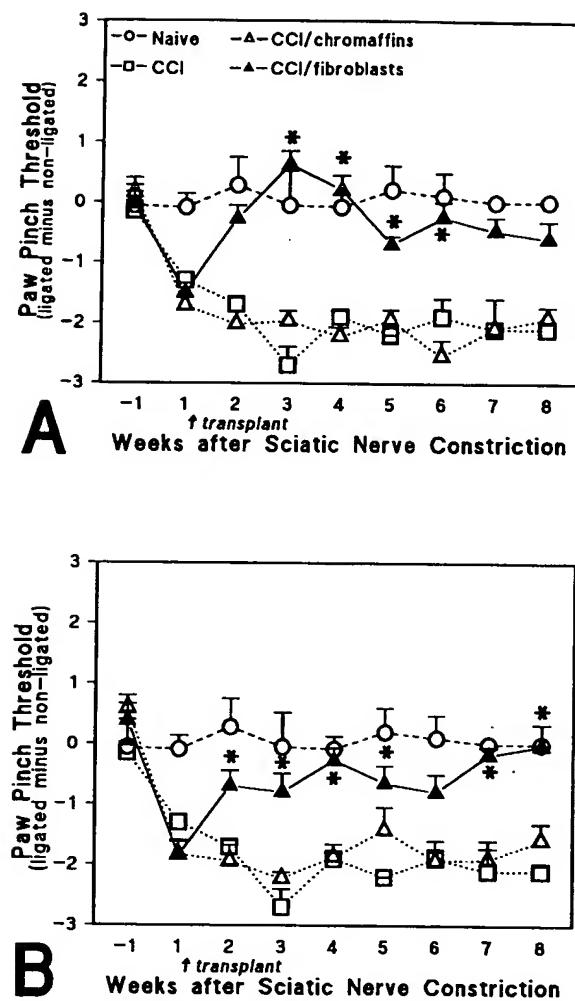


Figure 6. Tactile hyperalgesia after CCI and transplants. Animals were either left unoperated (○), given the CCI (□), or transplanted with rat RAD5.2 (A), bovine BADA20 (B) (▲), or rat (A) or bovine (B) fibroblast cells (Δ) 1 week following the CCI, 1 day following behavioral testing. Animals were tested for hindpaw withdrawal in a paw pinch device once every week for 1 week before and 8 weeks following CCI and before and after transplants. Only animals that demonstrated tactile hyperalgesia 1 week after CCI were transplanted. The data reported are the mean \pm SEM of the difference values for ligated paw minus the sham-operated paw of 14 animals in each group. Asterisks (*) indicate the chromaffin cell line transplants that differed significantly from the CCI condition at each time point: $p < 0.01$.

time point after transplant of rat chromaffin cells, the range of duration over 20 min in the ligated hindpaw was 2.5–10.0 s and 3.0–8.0 s for the bovine chromaffin transplanted animals. No cold plate sensitivity was observed in uninjured animals or on the sham-operated hindpaw (data not shown) of any animal in any test group. When rat and bovine fibroblast cells were transplanted after CCI, both the number and duration of hind-

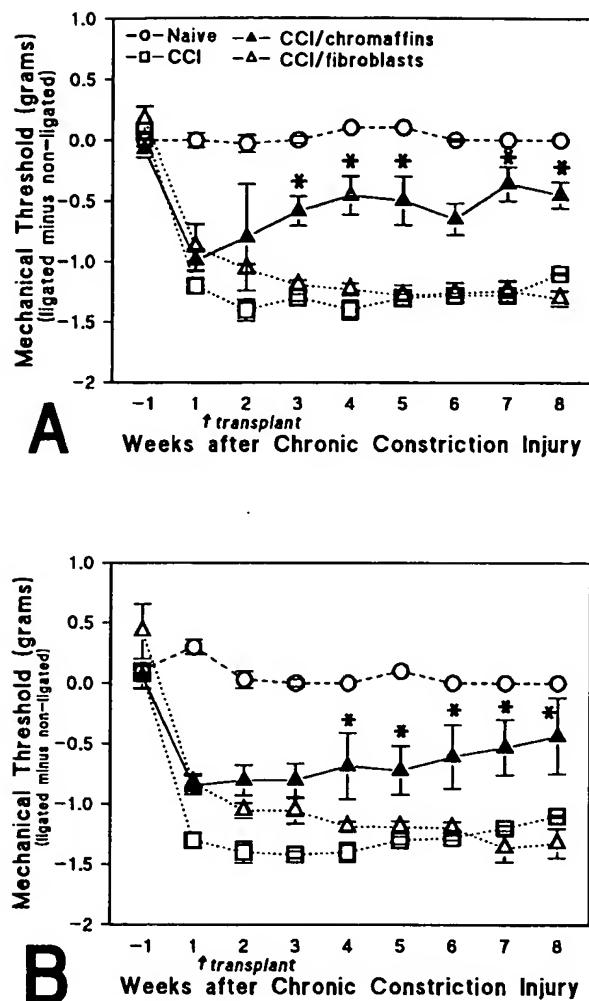


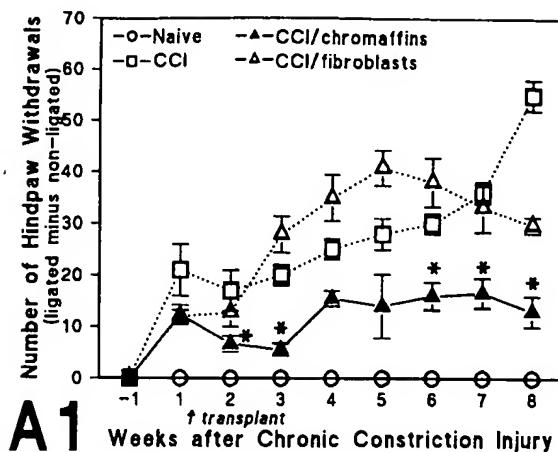
Figure 7. Tactile allodynia after CCI and transplants. Animals were either left unoperated (○), given the CCI (□), or transplanted with rat RAD5.2 (A), bovine BADA20 (B) (▲), or rat (A) or bovine (B) fibroblast cells (△) 1 week following the CCI, 1 day following behavioral testing. Animals were tested for hindpaw withdrawal to a graded series of von Frey hairs once every week for 1 week before and 8 weeks following CCI and before and after transplants. Only animals that demonstrated tactile allodynia 1 week after CCI were transplanted. The data reported are the mean \pm SEM of the difference scores for ligated paw minus the sham-operated paw of 14 animals in each group. Asterisks (*) indicate the chromaffin cell line transplants that differed significantly from the CCI condition at each time point: $p < 0.01$.

paw withdrawals were similar to CCI alone. The range of latency scores was 13–40 hindpaw lifts and 8.8–136.9-s duration over 20 min in the ligated hindpaw at 1 week posttransplant for the animals in Figure 8A1, and 11–49 hindpaw lifts and 26.8–200.2-s duration over 20 min in the ligated hindpaw for the animals in Figure 8A2.

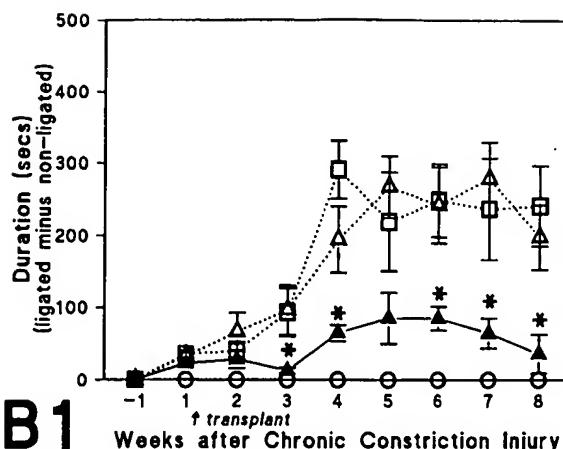
DISCUSSION

Transplants of primary chromaffin cells have been used in a variety of neurodegenerative diseases, including chronic pain. There is an extensive literature describing the use of primary adrenal tissue and allografts, as well as dissociated chromaffin cultured cells, to reverse the behavioral hypersensitivity related to chronic pain that develops after peripheral sciatic nerve constriction (13,28), hindpaw injection of formalin (72), laser-induced ischemia of the spinal cord (85), and excitotoxic chemical lesioning of the spinal cord (7). The use of chromaffin grafts and the changes in the spinal environment near the dorsal horn after transplant is best characterized with the use of the sciatic nerve constriction (CCI) model, where catecholamines and met-enkephalin synthesized and released by the chromaffin grafts (59, 60) are postulated to provide the antinociception in these models. However, the cell biology of these grafted chromaffin cells remains open to question, given the heterogeneity of cultured preparations from primary sources, as well as any deduction of the exact nature of possible antinociceptive molecules released by grafted cells in the various models of chronic neuropathic and neurogenic pain.

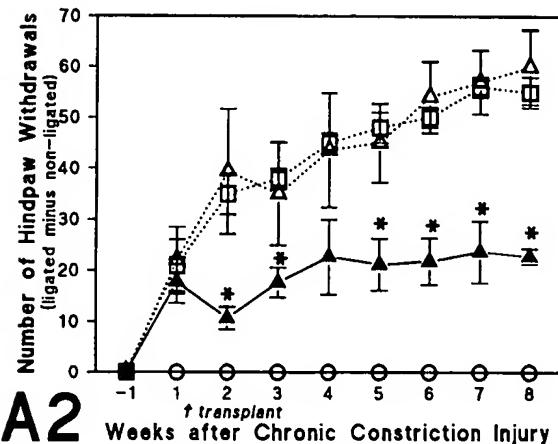
The initial characterization (16) of the rat and bovine chromaffin cell lines used here for transplant after conditional immortalization with the tsTag construct described the presence and regulation with glucocorticosteroids and cAMP activation of all the enzymes necessary for the identification of the chromaffin phenotype in vitro, using TH-, D β H-, and PNMT-ir in the cells as a marker for the noradrenergic and adrenergic phenotype of chromaffin cells. However, initial HPLC characterization of these immortalized chromaffin cells did not suggest that these cells are able to synthesize catecholamines in vitro. It has been thought that to alter spinal biochemistry for antinociception after chromaffin transplant requires that the chromaffin grafts be able to constitutively produce the catecholamine neurotransmitters during their survival near the spinal cord (57). Both the rat and bovine chromaffin cell lines express detectable TH and other catecholamine enzymes (16) when the cells are proliferating at permissive temperature in vitro, compared to the adrenal fibroblast control cell lines. The chromaffin cells also upregulate the TH protein expression during differentiation at nonpermissive temperature (39°C), when the mitotic drive from the large T antigen protein is off (22,39). It is possible that catecholamine synthesis may occur in vivo during differentiation in rat and bovine chromaffin cells after grafting, because the enzymes are expressed in surviving cells. Most surviving chromaffin grafted cells continue to contain TH-, D β H-, and PNMT-ir at 7 weeks after transplantation.



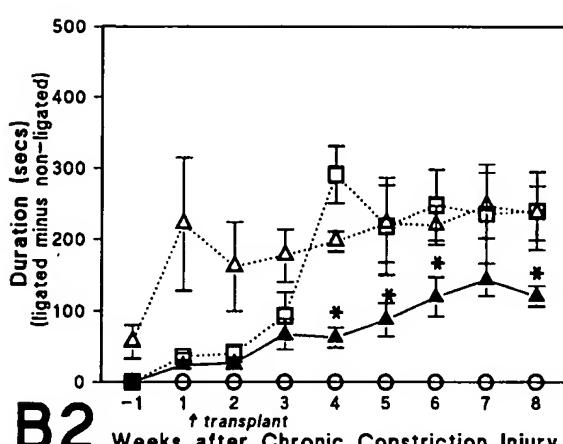
A1 Weeks after Chronic Constriction Injury



B1 Weeks after Chronic Constriction Injury



A2 Weeks after Chronic Constriction Injury



B2 Weeks after Chronic Constriction Injury

Figure 8. Cold allodynia after CCI and transplants. The number of hindpaw withdrawals (A1, A2) or the total duration of hindpaw lifting (B1, B2) in response to a cold plate over 20 min were counted once every week for 1 week before and 8 weeks following CCI and before and after transplants. Animals were either left unoperated (○), given the CCI (□), or transplanted with rat RAD5.2 (A1, B1), bovine BADA20 (A2, B2) (▲) chromaffin cells, or rat (A1, A2) or bovine (B1, B2) fibroblast cells (△) 1 week following the CCI, 1 day following behavioral testing. Only animals that demonstrated cold allodynia 1 week after CCI were transplanted. The data reported are the mean \pm SEM of the difference values for ligated paw minus the sham-operated paw of 14 animals in each group. ANOVA indicated that the data were statistically significant. Asterisks (*) indicate the chromaffin cell line transplants that differed significantly from the CCI condition at each time point: $p < 0.01$.

But it is not known at this time if grafts, which differentiate *in situ* on the pial membrane, also synthesize and release catecholamines. If chromaffin grafts do not make significant levels of catecholamines, then the antinociception the grafts provide may be a result of other antinociceptive molecules synthesized and released by the cells.

A number of other, possibly antinociceptive, molecules are synthesized by primary chromaffin tissue: namely, GAL, GABA, and 5-HT (8,32–34,37,55,78). Various other factors, such as chromogranins, the secretory acidic proteins, are synthesized by chromaffin cells as well (78). Histograin is found in high concentrations

in chromaffin granules (44), and may behave as an NMDA receptor antagonist. Recent studies indicate that an analog for the histograin peptide, Ser1-histograin, markedly attenuated nociception after CCI (71). These rat and bovine chromaffin grafts express many of these neurotransmitter and peptide immunoreactivities. The neurotransmitter GABA provided chronically by cell therapy (19), or acutely by intrathecal administration (18), is able to reverse neuropathic pain after nerve injury. Similarly, chronic delivery of 5-HT from cell grafts (15) provides antinociception after CCI. The peptide GAL, delivered into the intrathecal space after nerve injury, is also associated with antinociception (81). The

opioid ENK production by primary chromaffin grafts has been postulated as the most likely antinociceptive molecule synthesized by chromaffin cells (51). Increased CSF levels of ENK have been demonstrated with subarachnoid chromaffin grafts in rats (59) and humans (58). Transplants of chromaffin cell lines express ENK-ir (Figs. 4 and 5) *in vivo*, and treatment with glucocorticosteroids, such as dexamethasone, upregulates ENK expression in these same cell lines with differentiation *in vitro* (16). A similar scenario may take place *in vivo*, with glucocorticosteroids regulating the cellular maturity, survival, and expression of various antinociceptive molecules in the grafts.

Dexamethasone is known to decrease NGF mRNA and synthesis in many types of astrocytes and fibroblasts *in vitro* (35,48,49), while serum increases NGF production from fibroblasts (35). These studies suggest that the rat and bovine chromaffin cell lines that were proliferated in the presence of both dexamethasone and cocultured with fibroblasts were presumably inhibited from a switch to a neuronal phenotype. Because these cell lines might be dependent on other factors released from fibroblasts, they are not likely to be NGF dependent in the presence of dexamethasone and behave as mature chromaffin cells. Although the presence of corticoid receptors has not been assayed in these chromaffin cell lines, they are presumed to be present and functional, because the cells respond with increased enzyme expression when dexamethasone is included during the differentiation paradigm. How this *in vitro* alteration in adrenergic and noradrenergic phenotype with dexamethasone stimulation might affect transplanted conditionally immortalized chromaffin cell is not known, but presumably glucocorticoid levels are adequate in the CNS (14) to ensure a chromaffin phenotype in differentiated grafts.

A necessary criteria for successful grafting of immortalized chromaffin cell lines for the reversal of behavioral hypersensitivity after nerve injury is the actual chronic survival and expression of the chromaffin phenotype of grafts at the transplant location, the subarachnoid space, near the lumbar spinal cord, where the afferent fibers of the sciatic nerve enter the dorsal horn. Here, substantial numbers of the chromaffin cells survived at least 7 weeks after transplant into the spinal subarachnoid space, with the cells attached to the cord several segments, peripherally, rostral and caudal to the transplant site. With continued staining for TH-, D β H-, PNMT-, GABA-, ENK-, and 5-HT-ir in most of the surviving chromaffin cells throughout the experiment, it is possible or likely that the grafts may act as a supply for various inhibitory neurotransmitters, peptides, and opioids, or other molecules from the chromaffin "trophic cocktail" synthesized by adrenal medullary chromaffin cells (78), at least with a localized supply near the dorsal

horn and lumbar spinal cord. Although it is not known what the transplant time "window" might be that is required for successful reversal of chronic pain by cell grafts, the chromaffin grafts are placed at a similar time point after nerve injury as has been done with primary chromaffin cells. It is likely that even if nociceptive substances supplied by the grafts are low, it is sufficient to affect the local spinal biochemistry. It is interesting to note the apparent "hypoalgesia" induced by cell grafts in the measure of thermal and tactile hyperalgesia. There is no current explanation for such a phenomenon and all cell therapies after CCI, including primary chromaffin grafts, seem to induce this "overshoot." However, the affected animals do not behave abnormally or exhibit any paralysis in the hindlimb. Primary chromaffin cell grafts recover the endogenous dorsal horn GABA system after nerve injury and transplants, and there is no evidence that the endogenous spinal GABA system reaches anything but normal levels after chromaffin cell grafting (36). Returning the balance of inhibitory tone at the dorsal horn may directly, through release of antinociceptive substances from chromaffin grafts into the CSF, or indirectly, by modulating the ascending signal to higher supraspinal centers, recover the spinal systems. Alternative placement of grafts at intracerebral or intraventricular locations might significantly alter the rate of the reversal of nociception, and this strategy is currently being examined as well as analysis of the GABAergic content and changes in supraspinal centers after lumbar grafts of chromaffin cell lines after nerve and SCI.

A related issue for survival of chronic transplants with the substitution of immortalized for primary chromaffin cells is the use of xenogeneic bovine cells, such as the BADA.20 cell line used here. Primary bovine chromaffin cells that have been freed of endothelial and passenger, nonchromaffin, cells do not stimulate rat T-cell proliferative responses *in vitro* (12), do not require exogenous trophic factors for at least 8 weeks of robust survival in the rat subarachnoid location (47), and short-term immunosuppression after grafting in rats with cyclosporine A was sufficient for at least 1 year of survival and expression of the chromaffin cell phenotype (54). The bovine BADA.20 chromaffin graft demonstrated easily detectable cell numbers after 7 weeks, and such results are merely suggestive and likely to be an extreme underestimation of absolute cell numbers.

No animal in these studies developed tumors at the site of grafting over the 2-month period with the chromaffin cell lines containing the oncogenic Tag construct. The temperature-sensitive large T antigen used for immortalization rapidly degrades at nonpermissive temperature (38,39,76). Given the conclusions that the Tag protein expression, mRNA levels for Tag and mitotic drive is absent after a short differentiation time (16) *in vitro*,

and with the decrease of Tag-ir in chromaffin transplants after differentiation (Fig. 2), the immortalized chromaffin cell lines are not likely to be proliferative *in vivo*. Even if there is an initial cell division or two after grafting, the intensity of the BrdU-ir (from pretransplant cell treatment) does not diminish in the grafts, suggesting no dilution of signal (which would represent cell division in the grafts over time). As well, the total cell counts in the grafts at 8 weeks represents >3% of the 10^6 cells injected. These observations, along with the data that show the disappearance of Tag-ir over time after transplant *in vivo*, and as reported *in vitro* (16), strongly suggest that conditionally immortalized cell lines would be safe for use in humans. However, our laboratory is pursuing alternative immortalization/disimmortalization approaches to create stable cell lines for use with cell therapy to ensure that such an approach is practical. This technology involves excising the Tag oncogene before transplant, retaining and enhancing the cell phenotype (31). Others have concluded (39) that differentiation, at nonpermissive temperature, of tsTag-cells does not negatively alter the protein-synthesizing capacity from that of the cells grown at permissive temperature, and certainly that seems to be true of these chromaffin cell lines *in vitro* (16). Here, the grafted cell lines display a mature chromaffin phenotype, expressing many of the markers found in primary cultured and transplanted chromaffin cells.

Presumably, subarachnoid transplants of primary rat and xenogeneic bovine chromaffin cells reduce pain sensitivity after sciatic nerve injury through the release and diffusion of opioid peptides, and possibly catecholamines, from the grafts into regions of the CNS sites that modulate pain (60,65,66,79). The dorsal horn contains a high density of opiate receptors as well as enkephalineric interneurons (42), with intrathecal morphine eliciting potent analgesia (83). There is a high density of noradrenergic fibers in the spinal dorsal superficial laminae, and similar to morphine, intrathecal noradrenergic agonists produce analgesia (53), while the blockade of noradrenergic receptors in the spinal cord produces an increased sensitivity to noxious stimuli (64,67). Opioid peptides and catecholamines may act synergistically to induce their effects (82). With the simultaneous presence of both opioid-ir and inhibitory neurotransmitter enzyme-ir 7 weeks after grafting with nerve injury in both rat and bovine chromaffin transplants, it is possible that the grafts are synthesizing and, presumably, secreting or releasing one or both neuroactive substances throughout the time course of sensory testing.

In summary, immortalized chromaffin cell lines can be used as a potentially infinite homogeneous source for grafting and pain relief. The potential for tumor formation is not likely, with the decreased presence and activi-

ty of the Tag protein over 2 months after transplantation. Grafts are not rejected, even xenografts with short-term immunosuppression, and the chromaffin cell phenotype is expressed when cells are attached to the pial membrane in the subarachnoid space. This initial study provides evidence that conditionally immortalized primary cells used for cellular therapy can function as biologic "minipumps" after grafting and provide a model for the development of cell lines in the attenuation of chronic pain.

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